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The mitogen-activated protein kinase kinase 5 regulates the proliferation and biosynthetic processes in procyclic forms of *Trypanosoma brucei*

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The mitogen-activated protein kinase kinase 5 regulates the proliferation and biosynthetic processes in procyclic forms of *Trypanosoma brucei*

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Abstract

The pathogenic protozoan *T. brucei* alternates into distinct developmental stages in the mammalian and insect hosts. The mitogen-activated protein kinase (MAPK) signaling pathways transduce extracellular stimuli into a range of cellular responses, which ultimately lead to the adaptation to the external environment. Here, we combined a loss of function approach with stable isotope labelling with amino acids in cell culture (SILAC)-based mass spectrometry (MS) to investigate the role of the mitogen-activated kinase kinase 5 (MKK5) in *T. brucei*. The silencing of MKK5 significantly decreased the proliferation of procyclic forms of *T. brucei*. To shed light into the molecular alterations associated with this phenotype, we measured the total proteome and phosphoproteome of cells silenced for MKK5. In the total proteome, we observed a general decrease in proteins related to ribosome and translation as

well as down-regulation of several components of the fatty acid biosynthesis pathway. In addition, we observed alterations in the protein levels and phosphorylation of key metabolic enzymes, which point toward a suppression of the oxidative metabolism. Taken together, our findings show that the silencing of MKK5 alters cell growth, energy metabolism, protein and fatty acid biosynthesis in procyclic *T. brucei*.

Keywords

mitogen-activated protein kinase kinase 5 (MKK5), *T. brucei* , proteomics, phosphoproteomics, energy metabolism, fatty acids biosynthesis.

Introduction

The Kinetoplastida protozoan *Trypanosoma brucei* is the causative agent of African trypanosomiasis, also known as sleeping sickness. During its complex life cycle, *T. brucei* alternates into distinct developmental stages in the mammalian and insect hosts¹. In order to adjust to the different environment, major metabolic and morphological changes are required. In this context, post-translational modifications (e.g. protein phosphorylation) represent a dynamic mechanism that can mediate the prompt adaptation to the conditions encountered in each host, such as temperature, nutrient availability and pH². In fact, in trypanosomatids kinases belonging to the CMGC and STE groups, which comprise the mitogen-activated protein kinase (MAPK) signaling pathways, are overrepresented in comparison to humans³. The MAPK pathway transduce external signals into cellular responses, leading to changes in cell proliferation, survival and morphology, which ultimately accommodate the cellular physiology to the new environment⁴. In these intricate signaling cascades, a MAP kinase kinase kinase kinase (MKKKK, MAPKKKK,

MAP4K or STE20) phosphorylates and activates a MAP kinase kinase kinase (MKKK, MAPKKK, MAP3K or STE11), which in turn phosphorylates and activates a MAP kinase kinase (MKK, MAPKK, MAP2K or STE7) that then phosphorylates the conserved threonine and tyrosine residues located in the activation loop of a MAP kinase (MAPK)^{4,5}. In *T. brucei*, the MAPK pathway is composed by 2 MKKKK, 15 MKKK, 5 MKK and 15 MAPK³. To date, seven *T. brucei* MAPK have been studied and their involvement in a variety of cellular functions demonstrated. The MAPK KFR1 participates in the response to interferon-gamma in bloodstream forms⁶; MAPK2 is required for the differentiation into procyclic forms⁷; TbECK1 modulates cell growth in procyclic forms⁸; TbMAPK5 controls virulence and differentiation of bloodstream forms⁹; TbMAPK4 confers resistance to temperature stress in procyclic forms¹⁰, TbERK8 is required for cell growth in bloodstream forms¹¹ and MAPK1 is required for proliferation in procyclic forms¹². From the five MKK proteins of *T. brucei*, MKK1 and MKK5 have been previously studied. The knockout of these genes in bloodstream forms did not cause changes in cell growth in standard culture conditions; however, defects in proliferation were observed upon exposure to temperature stress¹³. Interestingly, in the same work, western blot analysis suggested that MKK5 expression is higher in the procyclic than in the bloodstream forms of *T. brucei*¹³, which may indicate that the role of this protein is developmental stage-specific. To date, the function of MKK5 in procyclic forms of *T. brucei* has not yet been addressed. The combination of quantitative proteomics and phosphoproteomics with perturbation of kinases and phosphatases is a powerful approach to discover the pathways and substrates regulated by these proteins at a systems level^{12,14-16}. Here, we combined a loss of function approach with SILAC-based proteomics and phosphoproteomics to uncover the role of MKK5 in procyclic

forms of *T. brucei*. Our results revealed that the silencing of MKK5 induces significant changes in the proliferation, in the total levels and phosphorylation of translation, fatty acid biosynthesis and energy metabolism-related proteins, thus indicating that this kinase is involved in the maintenance of homeostasis in procyclic forms of *T. brucei*.

Materials and Methods

MKK5 RNAi plasmid design, cloning and transfection

The DNA sequence of MKK5 (gene ID Tb927.10.5270 or Tb10.70.1800, Uniprot ID Q38B76) of *T. brucei brucei* TREU 927 was retrieved from GeneDB database. The selection of a specific target region for RNAi and the design of primers were performed using the web tool RNAit from TrypanoFAN using the default settings¹⁷. The resulting primers, MKK5 forward 5'-AAGTTCACAGGTCAAACCCG-3' and MKK5 reverse 5'-GTTCAGCAACAAGACCA-3', were used to amplify by PCR the RNAi target region from the genomic DNA of *T. brucei* Lister 427. The PCR reaction was performed using 1 U of Taq DNA polymerase high fidelity (Invitrogen), Platinum Taq buffer, 2 mM MgSO₄, 0.4 mM of each primer, 10 mM dNTPs and 100 ng of genomic DNA as template. The PCR was performed using one cycle of 94 °C during 2 minutes, followed by 30 cycles of 94 °C during 30 seconds, 55 °C for 30 seconds and 68 °C for 1 minute; a final extension cycle of 68 °C during 5 minutes was employed. Prior to cloning, the PCR products were purified using the High pure PCR product purification kit (Roche) following the manufacturer's instructions and run in a 0.8% agarose gel for confirmation of the amplicon size. The PCR product was cloned in a modified version of the p2T7-177 plasmid¹⁸, which contained the LacZ gene instead GFP. The LacZ cassette of the modified p2T7-177 plasmid was flanked by two *XcmI* restriction sites, providing 'T' overhangs after digestion with *XcmI*, which

are then cohesive with the ‘A’ extremities of the PCR products, thus allowing to use the TA cloning strategy. Moreover, the LacZ gene allows screening the positive colonies by color. For digestion, 2 µg of the plasmid, 10 U of *XcmI* (New England Biolabs), and NEB2 buffer were used, the reaction was incubated at 37 °C for 2h, followed by heat inactivation at 65 °C for 20 minutes. The digestion of the plasmid was confirmed using a 0.8% agarose gel and the corresponding band of the vector was excised and purified from the gel. The vector-insert ligation was performed using 1 U of T4 DNA ligase (Invitrogen), ligase buffer, 50 ng of the vector and 1:10 molar ratio vector-insert overnight at 16 °C. The resulting plasmids were transfected by heat shock into DH5α cells. Positive clones were screened by colony PCR and the RNAi target sequence was confirmed by DNA sequencing. From now on, the resulting RNAi MKK5-p2T7177 plasmid to silence MKK5 in *T. brucei* will be referred as MKK5 RNAi plasmid. Prior to transfection, 5 µg of the MKK5 RNAi plasmid was linearized using 8 U of *NotI* HF (New England Biolabs), NEB4 buffer, and BSA at 37 °C for 16 hours, followed by heat inactivation at 65 °C for 20 minutes. The linearized MKK5 RNAi plasmid was transfected by electroporation into procyclic forms of *T. brucei brucei* Lister 427 clone 29-13¹⁹. For transfection, 4 x 10⁷ cells were washed in electroporation buffer (129 mM NaCl, 1.5 mM KH₂PO₄, 8 mM KCl, 8 mM NaH₂PO₄, 1.5 mM MgCl₂, 0.09 mM CaCl₂, 2.4 mM CH₃COONa, pH 7.0), spin at 5,000 x g, 5 min at 4 °C and resuspended in 400 µl of the electroporation buffer in a 0.4 cm cuvette (BioRad). Then, 10 µg of linearized plasmid DNA was added to the cuvette and incubated on ice for 10 min. The cuvettes were subject to two pulses of electroporation (1.6 kV, 25 µF, and time constant of 0.8 s) in a Gene Pulse II Electroporation System (BioRad). The resulting transfected cells were cultured in the medium SDM-79 (LGC Biotecnologia)²⁰, supplemented with 10% FBS (Gibco), 15

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3 $\mu\text{g/ml}$ of G418 (Sigma) and 50 $\mu\text{g/ml}$ of hygromycin (Sigma) and incubated at 28 °C
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5 in 5% of CO₂. The selection was performed using 2.5 $\mu\text{g/ml}$ of phleomycin (Sigma).
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7 The transcription of the double-stranded RNAi to silence MKK5 is under control of
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9 two T7 promoters; to trigger the RNAi mechanism 2 $\mu\text{g/ml}$ of tetracycline (Sigma)
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11 were added in the first day, and 1 $\mu\text{g/ml}$ was added daily in the course of the
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13 experiments.
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15 16 RT-qPCR

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18 To determine MKK5 silencing efficiency we used RT-qPCR. Procyclic forms of *T.*
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20 *brucei* transfected with the MKK5 RNAi plasmid were treated (Tet+) or not (Tet-) with
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22 tetracycline for 3 and 4 days. The total RNA was isolated from 1×10^8 cells using
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24 RNeasy Kit (Qiagen) according to manufacturer's instructions. The cDNA synthesis
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26 was performed using 1 μg of RNA and 1 μM oligo dT, incubated for 10 min at 70 °C.
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28 Next, 2 μl of Improm-II buffer (Promega), 6 mM MgCl₂, 10 mM dNTPs, 20 U
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30 RNaseOUT (Life Technologies) and 2 μl Improm-II Reverse
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32 Transcriptase (Promega) were mixed in a final volume of 20 μl and incubated for 2 h
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34 at 42 °C. The resulting product was purified using Microcon YM-30 (Millipore) and
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36 resuspended in water at a concentration of 2 ng/ μl . The primers used to amplify
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38 MKK5 were designed using PrimerSelect in the DNASTAR software and their
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40 efficiency was assessed using a standard curve. The primers anneal at the 3'-UTR
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42 of MKK5 transcript, T_m 60 °C. In each RT-qPCR reaction, 0.5 μM of the forward 5'-
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44 GGTGTAGAACGACATGTGTATTTATTTAGGTG- 3' and reverse 5'-
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46 GTCCTCTCACAGTCCTTGCCCG- 3' primers was used, together with 10 ng of
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48 template and 10 μl of SYBR green (Life Technologies). For normalization, the
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50 expression levels of Actin, paraflagellar rod protein (PFR) and telomerase reverse
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52 transcriptase (TERT) were assessed²¹. The reactions were carried out in triplicates
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with appropriated non-targeting controls using the Applied Biosystems 7500 Real-Time PCR System. The analysis was performed using the average of the normalized MKK5 mRNA levels for each housekeeping gene and the remaining levels of MKK5 in the Tet⁺ cells expressed as a percentage of the MKK5 levels found in the Tet⁻ cells.

Growth curves

To investigate the impact of MKK5 silencing in the cell growth of procyclic forms of *T. brucei* we evaluated the growth rate of cells silenced or not for MKK5. For this, two distinct strategies were employed. In the first one, the cell density was assessed every day until cells reached the stationary growth phase using a Neubauer chamber. In the second strategy, cell density was measured every two days using a Z2 Coulter cell counter (Beckman Coulter), followed by a dilution of the original culture to 1 x 10⁶ cells/ml. In the second set-up, the cell growth was assessed for 10 days and cells were kept in exponential growth phase.

MS sample preparation

To accurately quantify the global changes induced by MKK5 silencing in *T. brucei* we used a SILAC-based approach^{12,22-24}. The SDM-79 medium without lysine and arginine was prepared in house²⁰. Procyclic forms of *T. brucei* transfected with the MKK5 RNAi plasmid were cultivated in SDM-79 SILAC medium supplemented with 10% 10 kDa dialysed FBS (Sigma), 15 µg/ml of G418 (Sigma) and 50 µg/ml of hygromycin (Sigma) either in the presence of ‘unlabelled’ lysine and arginine (Arg₀ and Lys₀) or in the presence of the ‘labelled’ counterparts (Arg₁₀ and Lys₄). In total, 18 mg/l of lysine and 53.75 mg/l of arginine were used. After incorporation of the labelled amino acids (4 days in culture in the SILAC medium), cells were treated with 2 µg/ml of tetracycline in the first day of RNAi induction and with 1 µg/ml of

tetracycline in the subsequent three days. Four days post induction, cells were harvested and the same number of MKK5 silenced cells (Tet+) was mixed to the control cells (Tet-). The experiments were performed in biological triplicates. The cells were washed twice with PBS and spin at 8,000 x g for 5 minutes at 4 °C. The resulting pellet was lysed in 8M urea buffer containing protease and phosphatase inhibitors (Thermo) and the pH was adjusted to 8. For reduction and alkylation, the protein mixture was treated with 1 mM DTT (Sigma) for 1h, room temperature; followed by 5.5 mM IAA (Sigma) treatment for 45 minutes, room temperature, protected from light. Subsequently, sample was diluted 1:6 in 50mM ABC (ammonium bicarbonate) and digested overnight with 2.5 µg of Trypsin (Promega). In the following day, the tryptic peptides were acidified to pH 2.5 with TFA (Sigma). For the analysis of the total proteome, 10 µg of the resulting peptides were desalted in C18 stage-tips^{25,26}. For the analysis of the phosphoproteome, TiO₂-based phospho-enrichment was performed. The TiO₂ beads (GL Sciences) were resuspended in TiO₂ buffer (30 mg/ml DHB, 80% ACN, 0.1% TFA) at a concentration of 500 µg/µl. The mixture was incubated for 10 min at 600 rpm. Subsequently, the beads were added to the peptides at a 5:1 ratio and incubated during 1h in rotor wheel at room temperature. Then, the mixture was spin at 3,000 rpm and the supernatant was collected and subjected to two additional rounds of incubation with TiO₂ beads. The phosphopeptides bound to the TiO₂ beads were resuspended in washing buffer I (30% ACN, 3% TFA), placed in a C8 stage-tip and spin at 2,600 rpm for 2 minutes. The wash with washing buffer I was repeated. Subsequently, three washes with washing buffer II (80% ACN, 0.3% TFA) were performed. Three sequential elution with 15% ammonium hydroxide in 40% ACN were performed.

Samples were speed vacuum dried to eliminate the ACN and resuspended in buffer A (0.5% acetic acid). Then, phosphopeptides were purified in C18 stage-tips^{25,26}.

MS data acquisition

The peptides were loaded onto an EASY-nLC system (Thermo Fisher Scientific) coupled online to the mass spectrometer Q-Exactive HF (Thermo Fisher Scientific). The chromatography was carried out in a 20 cm fused silica emitter, 75 μ m inner diameter (New Objective) packed in house with Reprosil Pur Basic 1.9 μ m (Dr. Maisch GmbH). For peptide elution, buffer A (0.1% formic acid) and Buffer B (80% ACN, 0.1% formic acid) were used. For the total proteome, a 250 min gradient of 300 nl/min with 2% to 30% buffer B was used. For each fraction of the phosphoproteome, a 120 min gradient of 300 nl/min with 2% to 42% buffer B was used. The eluting peptides were ionized and injected into the mass spectrometer via electrospray, spray voltage 2.1 kV, capillary heater 200 °C. The mass range for the full MS scan was 375-1,500 m/z with a resolution of 60,000 at 250 Th. From total proteome and phosphoproteome fractions, the top 15 and top 10 most intense ions, respectively, were isolated for higher-energy collision dissociation (HCD) fragmentation. The mass window for precursor ion selection was 1.4 m/z. The threshold for triggering MS2 was 1.8E5 for the total proteome and 1.0E4 for the phosphoproteome. The relative collision energy used was 27 and the mass resolution for the MS2 was 15,000. The singly charged ions were excluded and the ions that have been isolated for MS/MS were added to an exclusion list for 30 sec (total proteome) and 20 sec (phosphoproteome fractions). MS data were acquired using the Xcalibur software (Thermo Fisher Scientific) and raw data was processed using the MaxQuant computational platform.

MS data processing and analysis

MaxQuant software version 1.5.0.36 coupled to the Andromeda search engine^{27,28} were used to process the raw data with the following settings: Multiplicity 2 (labels Arg₁₀ and Lys₄); variable modifications Acetyl (Protein N-term), Oxidation (M) and Phospho (STY); fixed modification Carbamidomethyl (C); digestion mode Trypsin, maximum 2 missed cleavages. The re-quantify parameter was enabled. The curated reference proteome used for peptide identification was downloaded from Uniprot *Trypanosoma brucei* TREU 927 (8,587 entries). For identification, the false discovery rates (FDRs) at the protein and peptide level were set to 1%. For quantification, only unique peptides with minimum 2 ratio counts were used. The analysis of the data was done using Perseus version 1.5.2.11²⁹. Reverse peptides, potential contaminants and only identified by modification site (for the proteome) were removed. Only the class 1 phosphosites, as determined by localization probability ≥ 0.75 and score difference > 5 , were considered for the analysis. The normalized SILAC ratios from the MaxQuant tables of each biological replicate were \log_2 transformed and the intensities \log_{10} . For downstream analyses, we required that proteins and phosphosites were quantified in at least two out of three biological replicates. The up-regulated and down-regulated proteins were defined based on the Significance B statistical test (Benjamini-Hochberg FDR 5%)²⁷, which was calculated for each replicate separately. We considered as regulated proteins and phosphorylation sites significantly regulated in at least two out of three individual biological replicates and with standard deviation lower than the averaged SILAC ratio. To determine the Gene Ontology (GO) categories enriched among the subset of up-regulated and down-regulated proteins we used Fisher's test (Benjamini-Hochberg FDR 5%). For the protein-protein interaction analysis, we used the Uniprot IDs of the regulated proteins and phosphorylation sites for the search in STRING,

version 10.0, using the default settings³⁰. The output files were uploaded in Cytoscape Version 3.4.0 for visualization of the networks. The mass spectrometry proteomics and phosphoproteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD007910.

Results

The silencing of MKK5 is detrimental to the proliferation of procyclic forms of *T. brucei*

To investigate the role of MKK5 in procyclic forms of *T. brucei* we have used a stable inducible RNAi-based loss of function approach. For this, we selected a target region unique for MKK5 using the web tool RNAit¹⁷. This fragment was amplified by PCR and cloned into a modified version of the vector p2T7-177¹⁸. Procyclic forms of *T. brucei* were transfected by electroporation with the MKK5 RNAi plasmid. After selection, the MKK5 silencing was induced via addition of tetracycline in the cultures and cells were used for functional experiments (Figure 1 A).

To assess the silencing efficiency, we isolated the mRNA from cells treated or not with tetracycline for three and four days and the levels of remaining MKK5 transcripts were determined by RT-qPCR. At day three, the remaining levels of MKK5 were approximately half than those observed in control cells, whereas at day four, only 25% of MKK5 mRNA was still present in comparison to the control cells (Figure 1 B). Subsequently, to investigate the role of MKK5 in the proliferation of procyclic forms of *T. brucei*, we evaluated the growth rate of parasites silenced or not for MKK5 growing in SDM-79 supplemented with dialyzed serum using two different approaches. In the first one, we continuously measured the number of cells in a time

course until the parasites reached the stationary phase of growth. In the second set-up, parasites were maintained in exponential phase of growth and the cell density was measured every second day from day 2 to day 10 post-induction, which allowed to assess the effects of MKK5 silencing for longer. In both experimental conditions the silencing of MKK5 significantly decreased the growth rate of procyclic *T. brucei* (Figures 1C and 1D). We next investigated the growth of procyclic cells in SDM-79 supplemented with regular FBS, and also in this condition we observed a significant decrease in the growth rate (Figure 1E). To shed light into the molecular alterations underpinning the defects in cell proliferation upon MKK5 silencing we employed an unbiased SILAC-based MS approach. This workflow allows an accurate quantification of the proteins and phosphorylation sites modulated upon MKK5 knockdown (Figure 2).

SILAC medium

To replace the unlabeled lysine and arginine amino acids by the heavy isotope-labelled counterparts, we have prepared in house the SDM-79 medium without these amino acids. To determine whether the medium prepared in house was suitable for *T. brucei* culture, we compared the growth of *T. brucei* in our medium with the commercially available one from LGC. Our results show that the ability of *T. brucei* to grow in both commercial and in house prepared SDM-79 culture medium was indistinguishable (Supplementary figure 1A). Subsequently, to decrease the costs related to the labelled amino acids, we conduct growth tests using 50%, 25%, 12.5% of the original concentration of lysine and arginine. We observed that when we used 50% or 25% of the original concentration of lysine or arginine present in the conventional SDM-79 medium there was no significant differences in the growth of the parasites. We only observed proliferation defects when the concentration was

decreased to 12.5% or when lysine and arginine were completely removed (Supplementary figures 1B and 1C). Likewise, when using 25% of both lysine and arginine the proliferation of *T. brucei* was comparable to the counterpart cells growing in the medium containing the full amount of these amino acids (Supplementary figure 1D). Additionally, we tested the incorporation rate of labelled amino acids using 100%, 50% and 25% or the original concentration of lysine and arginine and we observed that the rate of labelled amino acids incorporation was efficient in the three conditions: more than 98% of the peptides were labelled after 4 days growing in the SILAC medium.

Global proteomics and phosphoproteomics analysis of MKK5 knockdown cells reveals alterations in translation, fatty acid biosynthesis and energy metabolism-related proteins and phosphorylation sites

In the SILAC-based total proteome of *T. brucei* silenced for MKK5, we unambiguously identified 3,965 proteins and accurately quantified 3,024 proteins in at least 2 out of 3 biological replicates (Supplementary Table 1). To determine in an unbiased manner alterations in the abundance of proteins belonging to specific gene ontology categories we used the 1D enrichment analysis³¹, and found that the abundance of proteins related to ribosome and translation was significantly reduced upon MKK5 silencing. These include the GOMF structural constituent of ribosome (p-value 6.73E-26), GOCC ribonucleoprotein complex (p-value 1.72E-24), GOBP translation (p-value 4.85E-24) and GOCC ribosome (p-value 8.29E-24) (Figure 3).

Subsequently, to ascertain the significantly up-regulated and down-regulated proteins upon MKK5 silencing we used the significance B test in each replicate separately (Benjamini-Hochberg FDR 0.05). In the supplementary table 1, we have indicated the regulated proteins and reported the FDRs for each individual replicate.

To determine the reproducibly regulated proteins, we required that the regulation occurred in at least two out of the three biological replicates and that the standard deviation was lower than the averaged SILAC ratio. Using this cut-off we found 11 proteins significantly up-regulated and 27 proteins down-regulated (Figure 4A and Table 1).

To determine the gene ontology (GO) categories and KEGG pathways overrepresented in the subset of regulated proteins, we conducted an enrichment analysis. Among the up-regulated proteins, the KEGG pathway TCA cycle was enriched (Figure 4B). In the subset of down-regulated proteins, GO categories and KEGG pathways related to fatty acid biosynthesis, endoplasmic reticulum (ER), carboxylic acid metabolic process and transferase activity were enriched, thus indicating these functions were reduced in procyclic cells silenced for MKK5 (Figure 4C).

Next, to determine the changes induced by the silencing of MKK5 in the phosphoproteome of *T. brucei* and therefore to gain insights regarding the potential targets of this kinase, we used a sequential TiO₂-based enrichment coupled to SILAC-based MS. Using 250 µg of starting material and three sequential TiO₂ incubations, we identified 1,482 class 1 phosphosites: 1,267 on serine residues (85.5%), 208 on threonine (14.0%) and 7 on tyrosine (0.05%). The 914 phosphosites quantified in at least 2 out of 3 biological replicates were used for downstream analyses (Supplementary table 2). We normalized the levels of the phosphorylation sites to the total levels of the corresponding protein when it was quantified in the total proteome. For those sites where the total levels of the corresponding protein were not quantified we maintained the SILAC ratio value for the phosphorylation site. In total, 88.7% of the phosphorylation sites could be normalized and are indicated in

the Supplementary table 2. Then, to determine the significantly and reproducibly regulated phosphorylation sites we used the same criteria previously described for the total proteome. This analysis resulted in 24 phosphorylation sites significantly up-regulated and 8 significantly down-regulated (Table 2). The most up-regulated and down-regulated phosphorylation sites, respectively, were on the serine 284 of the alpha subunit of pyruvate dehydrogenase E1 (PDHE1 α) and on the serine 207 of the delta subunit of the eukaryotic translation initiation factor 2B (eIF2B δ) (Supplementary figure 2). Interestingly, these residues are conserved with the corresponding human proteins (Supplementary figures 3 and 4). Moreover, proteins that can influence rearrangements of the cytoskeleton, intracellular trafficking and cell migration were differentially phosphorylated. This was the case for the up-regulated phosphorylation sites on serine 95 of beta tubulin (Q4GYY6), threonine 757 of intraflagellar transport protein IFT88 (Q386Y0), serine 2,053 of the microtubule-associated protein (Q389U9) and the down-regulated site on the serine 469 of kinesin-like protein (Q38CW6). Additionally, ubiquitin-related proteins had phosphorylation levels increased upon MKK5 silencing, these sites were on the serine 2 of ubiquitin-fold modifier 1 (Q57UL0), serine 9 of ubiquitin carboxyl-terminal hydrolase (Q385P1) and serine 184 of ubiquitin carboxyl-terminal hydrolase (Q583R5).

Finally, in order to integrate the proteomic and phosphoproteomic data and to gain insights regarding the functional relationship among the regulated proteins and phosphorylation sites we used STRING. In the network generated from this analysis, we can observe that several mitochondrial proteins involved in energy metabolism were linked. Additionally, components of the fatty acid biosynthesis pathway and cytoskeleton/cell motility-related proteins were connected (Figure 5).

Discussion

Here, we show for the first time the involvement of the protein MKK5 in cell growth, energy metabolism, translation and fatty acid biosynthesis in procyclic forms of *T. brucei*.

Using different conditions to assess *T. brucei* the cell growth we found that the silencing of MKK5 significantly decreased the proliferation of procyclic cells. The effects on cell growth observed in our study were more pronounced when the SDM-79 medium was supplemented with dialyzed FBS, with more than 30% decrease in cell number in comparison to 15% decrease in the presence of regular FBS. This finding may suggest that either low molecular weight soluble factors or free amino acids found in the regular FBS can function as upstream regulators of this pathway. This observation is particularly relevant considering that procyclic *T. brucei* exist in the insect midgut, an environment deprived of glucose, where the energetic needs are met via amino acids catabolism. In the literature, the knockout of MKK5 in bloodstream forms did not alter the cell growth¹³, thus indicating that the requirement for this kinase is likely developmental stage-specific.

The global SILAC-based approach employed in our study provided valuable insights regarding the role of MKK5. First, using the 1D analysis, we observed a general decrease in proteins related to ribosome and translation. Interestingly, the most down-regulated phosphorylation site quantified in our study was on the delta subunit of the eukaryotic translation initiation factor 2B (eIF2B δ), which is part of the regulatory subcomplex of the holoenzyme. eIF2B is a multi-subunit protein critical for the regulation of the initiation of protein synthesis and its activity is inhibited in response to different types of stress^{32,33}. The precise role of the phosphorylation site

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we found regulated on the activity of eIF2B complex is still unknown. One hypothesis is that the modulation of this site may regulate protein synthesis initiation and be linked to the general decrease in the abundance of proteins related to translation observed upon MKK5 silencing. As a future step to characterize this further, mutagenesis of this residue coupled to protein synthesis profiling strategies could be employed.

Another interesting finding of our study corresponds to the down-regulation of several components of the fatty acid biosynthesis pathway. *T. brucei* exploits the endoplasmic reticulum (ER)-based elongase (ELO) pathway for fatty acid biosynthesis³⁴. In this pathway, the enzymes elongases (ELO1-3), which are integral membrane proteins residents in the ER, extend the fatty acid chains: ELO1 extends C4 to C10, ELO2 extends C10 to C14, and ELO3 extends C14 to C18³⁵. Here, we found that the knockdown of MKK5 in *T. brucei* decreased the protein levels of the 2 elongases (Q57UP8, Q57UP6); 2 desaturases (Q587G0 and Q57YK1), and of the fatty acyl CoA synthetase 2 (Q38FB9). In addition, two other enzymes that provide acetyl-CoA for lipid biosynthesis were down-regulated upon MKK5 silencing: carnitine O-acetyltransferase (Q386T6) and acetyl-coenzyme A synthetase (Q57XD7)³⁶. Because MKK5 silencing alters the protein levels of different components of the fatty acid biosynthesis pathway, this kinase could ultimately impact key cellular functions, such as energy storage, composition and fluidity of the plasma membrane.

In addition, the silencing of MKK5 altered the total levels and phosphorylation of proteins involved in energy metabolism. While bloodstream forms of *T. brucei* produce ATP via glycolysis, the procyclic forms are more flexible; they are able to adapt to distinct carbon sources and to produce energy using both the glycolytic and

oxidative pathways^{37,38}. In the subset of up-regulated proteins, the KEGG pathway tricarboxylic acid (TCA) cycle was enriched and metabolic enzymes of the TCA cycle were linked in the STRING-based network. The enzyme citrate synthase (Q388Q5) that catalyzes the conversion of acetyl-CoA and oxaloacetate into citrate, the first reaction of the TCA cycle; and the enzyme isocitrate dehydrogenase (Q387G0), that catalyzes the oxidative decarboxylation of isocitrate, producing alpha-ketoglutarate, CO₂ and NADH, were up-regulated. Of note, procyclic *T. brucei* can use parts of the TCA cycle for different purposes, such as for the degradation of proline and glutamate into succinate, generation of malate for gluconeogenesis and transport of acetyl-CoA from the mitochondria to the cytosol for fatty acids biosynthesis³⁹. In fact, it has been proposed that the acetyl-CoA produced in the mitochondria can be exchanged to the cytoplasm via citrate and then used for lipid synthesis⁴⁰. Moreover, we observed an up-regulation of Succinyl-CoA:3-ketoacid-coenzyme A transferase (ASCT), which converts acetyl-CoA into acetate. In this pathway, the acetate production occurs in two steps: first, ASCT transfers the CoA of acetyl-CoA to succinate, generating acetate and succinyl-CoA, which is then converted into succinate by the enzyme succinyl-CoA synthetase⁴¹. Noteworthy, the ASCT pathway also produces ATP and together with the TCA cycle and the respiratory chain comprise the three mitochondrial pathways generating ATP in the insect-stage *T. brucei*^{37,38}. Intriguingly, the most up-regulated phosphorylation site detected in response to MKK5 silencing was on the alpha subunit of pyruvate dehydrogenase E1 (PDHE1 α), which catalyzes a rate-limiting step in the conversion of pyruvate into acetyl-CoA. PDHE1 α is part of the pyruvate dehydrogenase complex that links glycolysis to the TCA cycle. In procyclic cells, a decreased growth rate was observed in cells silenced for PDHE1 α ³⁷. In humans, the enzymatic activity of the pyruvate

dehydrogenase complex is inhibited via phosphorylation of any of the three sites of PDHE1 α : serine 232, serine 293, and serine 300⁴². Using multiple sequence alignment, we found that the up-regulated phosphorylation site at serine 284 of *T. brucei* PDHE1 α aligns with the serine 293 of the human PDHE1 α , and out of the three regulatory sites of the human enzyme, this is the only one conserved with the *T. brucei* protein. In fact, a previous report that compared the sequence of the human PDHE1 α with representative species from other taxonomic groups, reported one phosphosite for *T. brucei*⁴³. If this phosphorylation site has also an inhibitory function in the pyruvate dehydrogenase complex of *T. brucei* as it does in the human, we can anticipate a decreased production of acetyl-CoA from pyruvate, which in turn could impact both ATP production and lipid biosynthesis. Additionally, among the down-regulated proteins we found succinate dehydrogenase (Q38EW9), an enzyme of the Complex II of the respiratory chain; and, an electron transfer protein (Q57YX5). The down-regulation of components of the respiratory chain could indicate an overall decrease in the production of ATP using this route. Overall, our data suggests that MKK5 silencing affects the metabolic homeostasis of the insect-stage *T. brucei* by regulating the total levels and phosphorylation of several metabolic enzymes. As a future step to unravel the consequences of MKK5 silencing in the overall energy production, metabolomics and tracing studies could prove informative.

The changes in phosphorylation measured in our study are likely an indirect effect of MKK5 knockdown, since we did not detect regulated phosphorylation sites within the activation loop of MAP kinases, which are the canonical targets of MKK proteins. We found the threonine and tyrosine residues located in the activation loop of one MAPK (Q381A7) phosphorylated in this study; however, these sites were not regulated

upon MKK5 silencing. The fact we did not identify multiple MAPK phosphorylated may reflect the amount of starting material used in our study, which was 250 µg. Previous phosphoproteomic studies performed in *T. brucei* that used from 2.5-10 mg of starting material have identified from 4 to 9 MAPKs phosphorylated^{12,24,44}. To date, in trypanosomatids, two MKK-MAPK pairs have been identified. The orthologue of *T. brucei* MKK5 in *Leishmania mexicana* has been shown to phosphorylate the MAPK LmxMPK4 *in vitro*⁴⁵. Moreover, in *L. major*, MKK1 has been shown to phosphorylate MPK3 and these kinases regulate the flagellar length⁴⁶. To unravel the relationship among the components of the MAPK pathway in *T. brucei*, different protein-protein interaction (PPI)-based approaches could prove informative, such as yeast two-hybrid, co-localization assays or pull-down analyses.

Conclusion

We have shown for the first time that MKK5 influences the proliferation of procyclic forms of *T. brucei*. Furthermore, we have quantified the molecular alterations at the protein and phosphorylation levels upon MKK5 silencing using a SILAC-based MS approach. Our work provides a global overview of MKK5-responsive pathways and phosphorylation sites, which can be used as a valuable resource to further explore the role of this protein. Our findings indicate that MKK5 regulates cell growth, protein translation, fatty acids biosynthesis and energy metabolism-related proteins and phosphosites. Collectively, these findings can open new avenues to be explored in future work to dissect the mechanistic role of this kinase.

Supporting information

Supplementary figure 1: Comparison of the growth of procyclic *T. brucei* in the commercial SDM-79 from LGC with the SDM-79 produced in house and evaluation of cell growth using decreasing concentration of lysine (K) and arginine (R).

Supplementary figure 2: Annotated spectra of the most regulated phosphorylation sites on eIF2B δ and PDHE1 α .

Supplementary figure 3: The phosphorylation site on serine 207 of *T. brucei* eIF2B δ (Q4FKA6) aligns with the serine 181 of human eIF2B δ (Q9UI10).

Supplementary figure 4: The phosphorylation site on serine 284 of *T. brucei* PDHE1 α (Q388X3) aligns with the serine 293 of human PDHE1 α (P08559).

Supplementary table 1: Total proteome of *T. brucei* silenced for MKK5. Table report the list of quantified proteins with their corresponding IDs, SILAC ratios, and FDR values.

Supplementary table 2: Phosphoproteome of *T. brucei* silenced for MKK5. Table report the list of quantified phosphorylation sites with their corresponding IDs, SILAC ratios, and FDR values.

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Conflict of interest

The authors declare no conflict of interest.

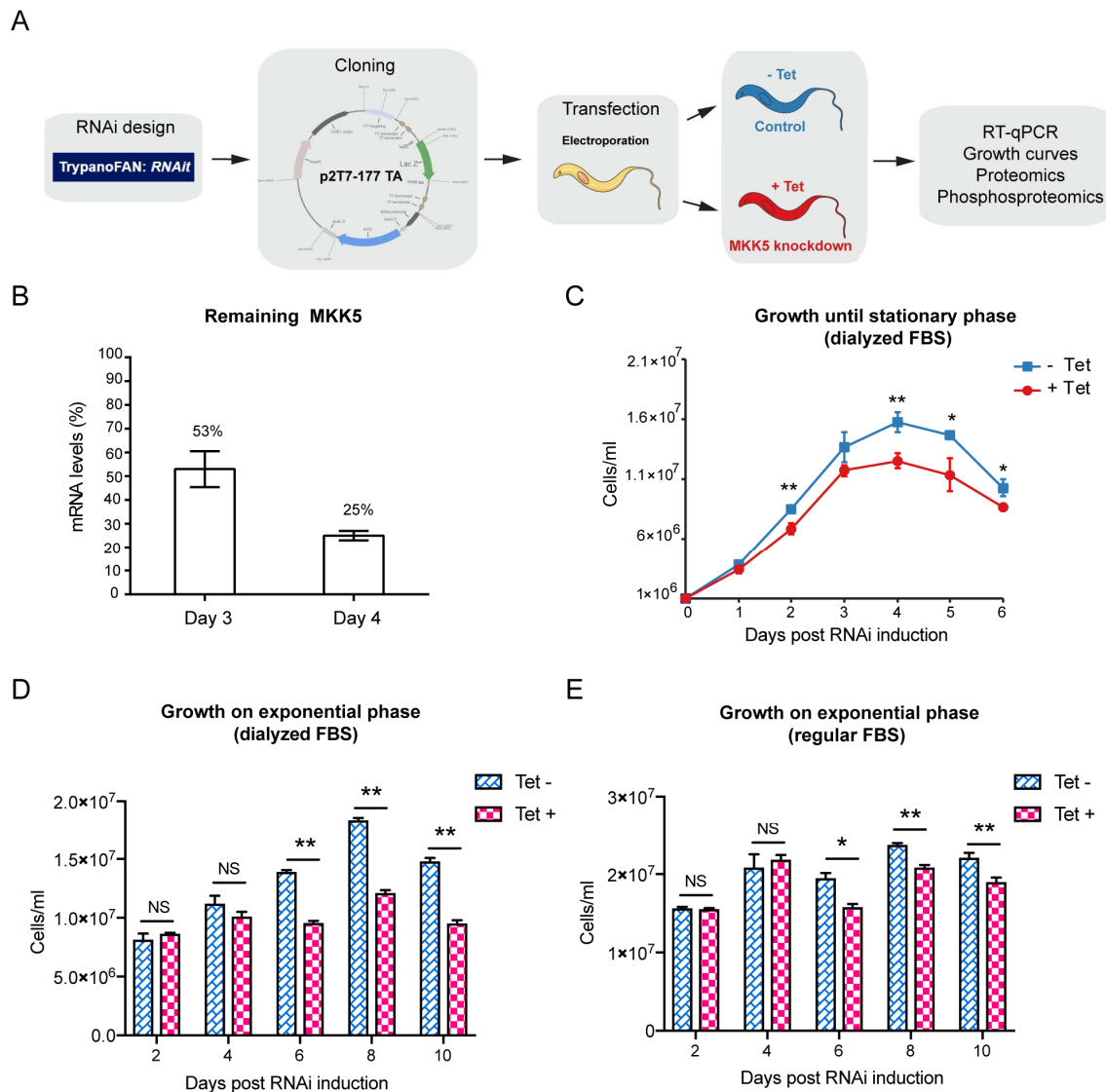


Figure 1: The silencing of MKK5 is detrimental for the growth of procyclic forms of *T. brucei*. (A) Experimental workflow used for RNAi construct design, cloning and transfection. Trypanosoma image adapted from medical servier art. (B) MKK5 silencing efficiency determined by RT-qPCR. The results are expressed as a percentage in comparison to control cells. mRNA levels were normalized to Actin, PFR and TERT. (C) Growth curve until stationary phase in dialyzed FBS. (D) Long-term growth curve in dialyzed FBS. (E) Long-term growth curve in regular FBS.

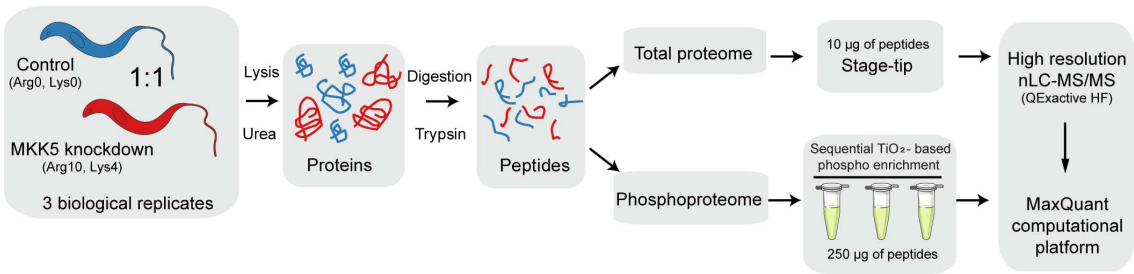


Figure 2: SILAC-based MS proteomics and phosphoproteomics workflow. *T. brucei* were SILAC labelled and the knockdown of MKK5 was induced via addition of tetracycline into the cultures for four days. Then, the same number of induced and non-induced cells was mixed, cells were lysed and proteins digested into peptides. For the analysis of the total proteome, peptides were purified in stage-tips and then run in the mass spectrometer. For the analysis of the phosphoproteome, the phosphopeptides were enriched using three sequential incubations with TiO₂ and then run in the mass spectrometer QExactive HF. All experiments were performed in biological triplicates and the raw data was processed and analyzed using the MaxQuant computational platform. Trypanosoma image adapted from medical servier art.

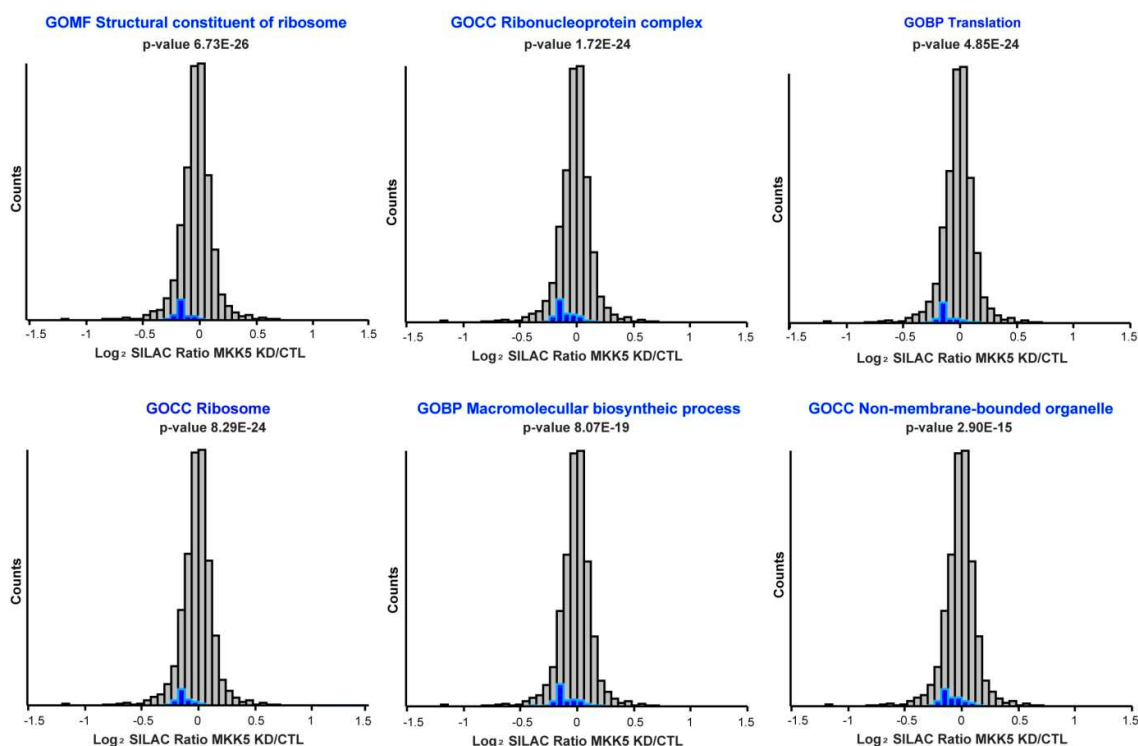


Figure 3: The unbiased 1D enrichment analysis reveals the abundance of proteins related to ribosome and translation is decreased upon MKK5 silencing. The histograms show in grey the global distribution of the quantified proteins. Highlighted in blue are the proteins belonging to the respective GO categories. The threshold used was 0.01 Benjamini-Hochberg FDR and the p-values are indicated.

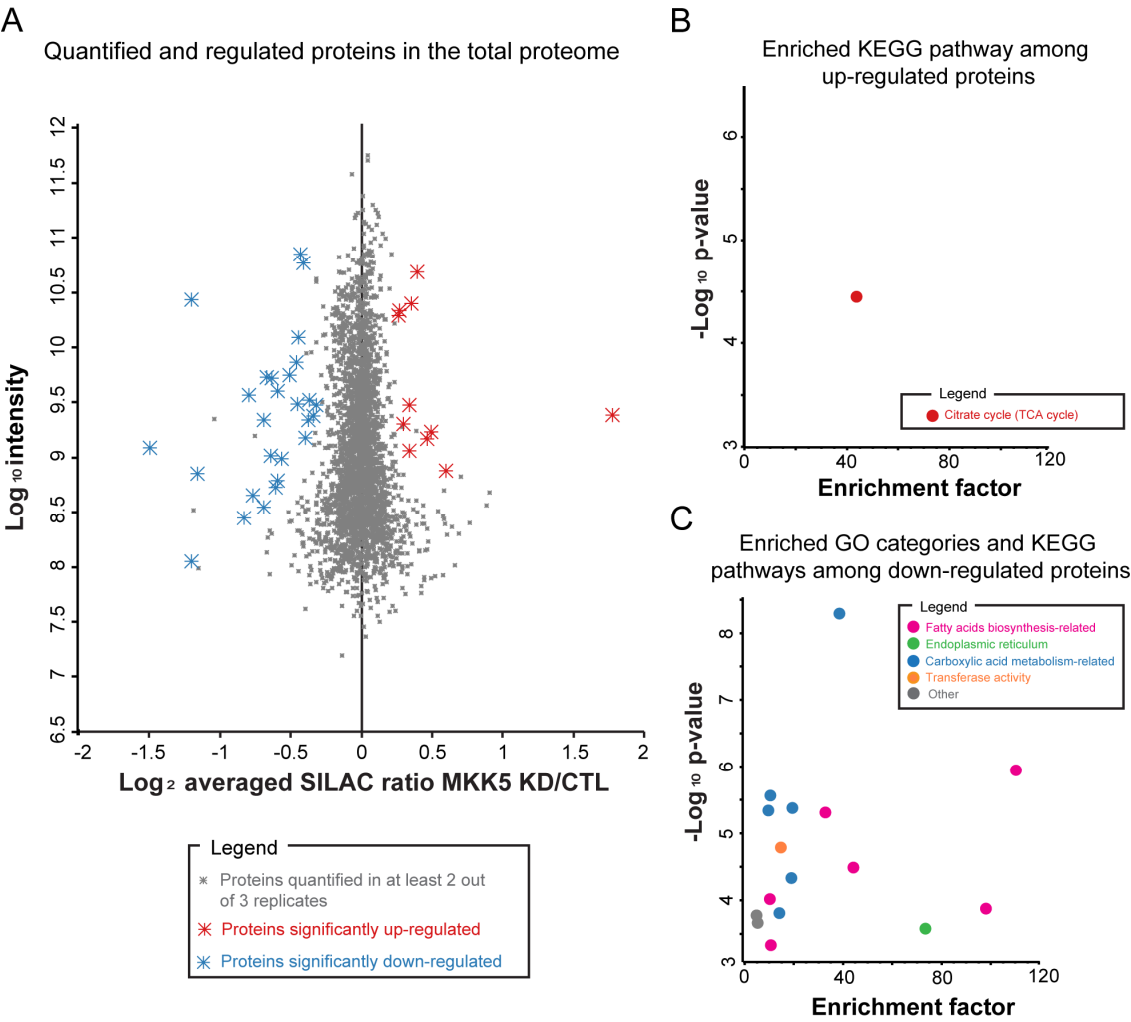


Figure 4: The silencing of MKK5 alters the total levels of proteins related to fatty acids biosynthesis and energy metabolism. (A) Scatter plot showing the quantified proteins, highlighted in red and blue are the proteins significantly regulated in at least two of three replicates determined by Significance B (0.05 Benjamini-Hochberg FDR) and with low variability across replicates. (B) Enrichment analysis of the GO categories and KEGG pathways in the subset of significantly up-regulated and (C) down-regulated proteins determined by Fisher's test (0.05 Benjamini-Hochberg FDR).

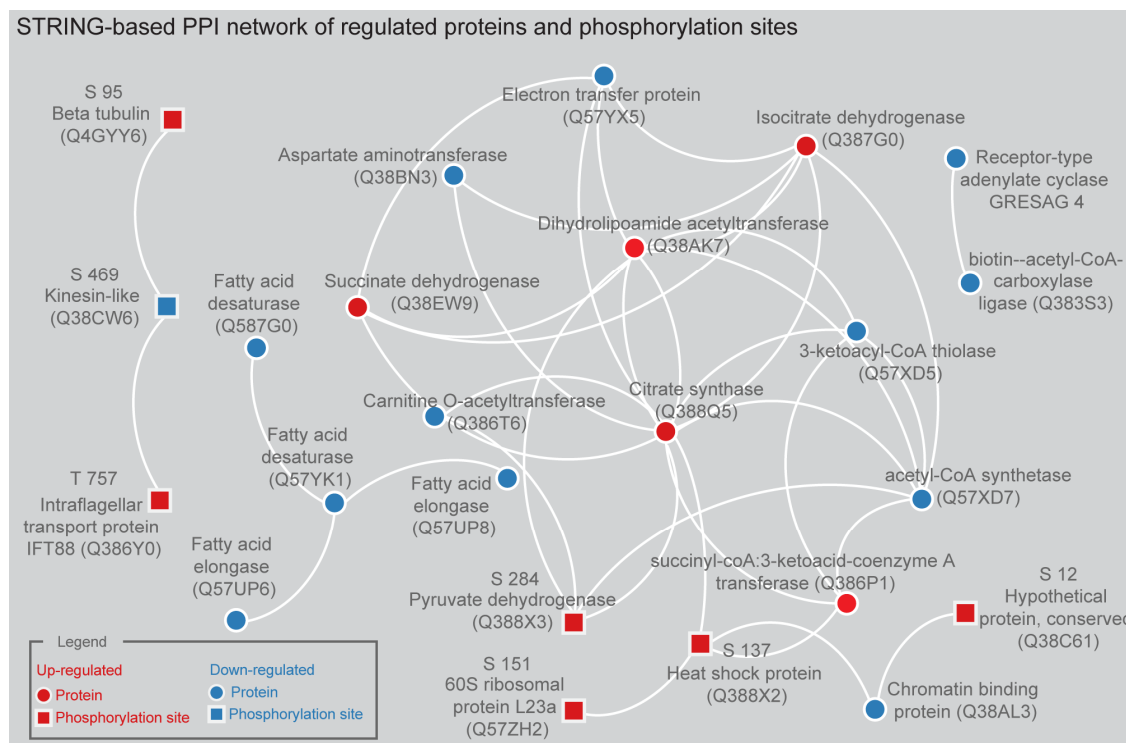


Figure 5: Functional relationship among the regulated proteins and phosphorylation sites determined by STRING. Modified residues are listed above each regulated phosphorylation sites.

Uniprot ID	Gene ID	Protein name	Log ₂ SILAC Ratio			Average	SD
			Rep 1	Rep 2	Rep 3		
Q57XQ8	Tb927.7.2160	Uncharacterized protein	1.864	1.831	1.628	1.775	0.128
Q57VC7	Tb927.5.1360	Uncharacterized protein	0.619	1.018	0.163	0.600	0.428
Q57TY7	Tb927.7.7500	Iron/ascorbate oxidoreductase family protein, putative	0.590	0.518	0.380	0.496	0.107
Q586R6	Tb927.2.4700	Uncharacterized protein	0.571	-	0.364	0.467	0.146
Q388Q5	Tb10.05.0150	Citrate synthase	0.386	0.392	0.420	0.399	0.018
Q386P1	Tb11.02.0290	Succinyl-CoA:3-ketoacid-coenzyme A transferase	0.360	0.315	0.379	0.351	0.033
Q57WY9	Tb927.3.1080	Uncharacterized protein	0.362	0.291	0.375	0.343	0.045
Q587H4	Tb927.2.2770	Uncharacterized protein	0.278	0.356	0.386	0.340	0.056
Q38EV9	Tb09.160.4480	Putative uncharacterized protein	0.239	0.337	0.309	0.295	0.050
Q38AK7	Tb10.6k15.3080	Dihydrolipoamide acetyltransferase, putative	0.263	0.303	0.246	0.271	0.030
Q387G0	Tb11.03.0230	Isocitrate dehydrogenase [NADP]	0.271	0.252	0.265	0.263	0.010
Q57Y77	Tb927.7.2670	Uncharacterized protein	-0.288	-0.294	-0.373	-0.318	0.047
Q584Q7	Tb927.6.2490	Uncharacterized protein	-0.347	-0.358	-0.314	-0.340	0.023
Q57XD7	Tb927.8.2520	Acetyl-coenzyme A synthetase	-0.364	-0.353	-0.390	-0.369	0.019
Q38BN3	Tb10.70.3710	Aspartate aminotransferase	-0.376	-0.276	-0.471	-0.374	0.098
Q57WH4	Tb927.7.3590	Uncharacterized protein	-0.326	-0.485	-0.375	-0.395	0.082
Q57XD5	Tb927.8.2540	3-ketoacyl-CoA thiolase, putative	-0.406	-0.435	-0.396	-0.412	0.020
Q57YA8	Tb927.7.2980	Uncharacterized protein	-0.386	-0.468	-0.438	-0.431	0.042
Q383S3	Tb11.01.1820	Biotin--acetyl-CoA-carboxylase ligase	-0.419	-0.439	-0.474	-0.444	0.028
Q389R7	Tb10.406.0290	Protein tyrosine phosphatase, putative	-0.502	-0.466	-0.394	-0.454	0.055
Q57UP8	Tb927.7.4180	Elongation of fatty acids protein	-0.447	-0.488	-0.445	-0.460	0.024
Q57X18	Tb927.8.5640	Uncharacterized protein	-0.463	-0.539	-0.516	-0.506	0.039
Q381P0	Tb11.01.7095	Putative uncharacterized protein	-0.535	-0.558	-0.609	-0.567	0.038
Q57YX5	Tb927.8.3380	Electron transfer protein, putative	-0.570	-0.598	-0.610	-0.593	0.020
Q587G0	Tb927.2.3080	Fatty acid desaturase, putative	-0.486	-0.712	-0.582	-0.593	0.113
Q57UP6	Tb927.7.4160	Elongation of fatty acids protein	-0.594	-	-0.620	-0.607	0.019
Q38FB9	Tb09.160.2780	Fatty acyl CoA synthetase 2	-0.609	-0.622	-0.673	-0.634	0.034
Q57VY1	Tb927.5.320	Receptor-type adenylate cyclase GRESAG 4	-0.847	-0.487	-0.586	-0.640	0.186
Q38EW9	Tb09.160.4380	Succinate dehydrogenase, putative	-0.641	-0.737	-0.630	-0.670	0.059
Q57ZJ0	Tb927.7.4990	Uncharacterized protein	-0.944	0.088	-1.207	-0.688	0.685
Q386T6	Tb11.18.0006	Carnitine O-acetyltransferase, putative	-0.699	-0.663	-0.702	-0.688	0.022
Q580N2	Tb927.3.4100	Uncharacterized protein	-0.854	-0.799	-0.648	-0.767	0.107
Q38A66	Tb10.6k15.1350	Pteridine transporter, putative	-0.719	-0.911	-0.753	-0.795	0.102
Q38AL3	Tb10.6k15.3150	Chromatin binding protein, putative	-	-1.024	-0.636	-0.830	0.274
D6XIG3	Tb927.6.4340	U6 snRNA-associated Sm-like protein	-0.426	-1.714	-1.347	-1.162	0.664
Q385F9	Tb11.02.3920	TatD related deoxyribonuclease, putative	-	-1.345	-1.051	-1.198	0.208
Q57VW9	Tb927.5.440	Trans sialidase, putative	-1.152	-1.285	-1.167	-1.201	0.073
Q57YK1	Tb927.8.6000	Fatty acid desaturase, putative	-1.400	-1.648	-1.429	-1.493	0.136

Table 1: Significantly regulated proteins upon MKK5 silencing in the total proteome.

Uniprot ID	Gene ID	Protein name	Site	Log ₂ SILAC Ratio			Average	SD
				Rep 1	Rep 2	Rep 3		
Q388X3	Tb10.389.0890	Pyruvate dehydrogenase E1 component alpha subunit, putative	S 284	1.347	1.467	1.351	1.388	0.068
Q387B6	Tb11.47.0013	Putative uncharacterized protein	S 482	1.090	1.190	1.272	1.184	0.091
Q584N4	Tb927.2.240	Retrotransposon hot spot (RHS) protein, putative	S 275	1.185	1.044	-	1.114	0.100
Q4GYY6	Tb927.1.2330	Beta tubulin	S 95	0.631	1.449	-	1.040	0.579
Q384X8	Tb11.02.4760	Putative uncharacterized protein	S 340	0.751	1.325	-	1.038	0.406
Q385G4	Tb11.02.3860	Putative uncharacterized protein	S 410	1.068	0.906	0.957	0.977	0.083
Q38C61	Tb10.70.6035	Putative uncharacterized protein	S 12	1.310	1.128	0.467	0.968	0.443
Q385Z7	Tb11.02.1680	Lectin, putative	S 454	0.882	0.925	1.002	0.936	0.061
Q57UL0	Tb927.8.5380	Ubiquitin-fold modifier 1	S 2	0.841	0.887	0.801	0.843	0.043
Q386Y0	Tb11.55.0006	Intraflagellar transport protein IFT88, putative	T 757	0.962	0.744	0.810	0.839	0.112
Q38F06	Tb09.160.3990	Cation transporter, putative	S 2	0.473	0.897	1.037	0.802	0.294
Q385B5	Tb11.02.4390	Putative uncharacterized protein	S 434	0.777	0.782	-	0.780	0.003
Q38F06	Tb09.160.3990	Cation transporter, putative	S 4	0.993	-	0.466	0.730	0.373
Q389D9	Tb10.389.1760	Putative uncharacterized protein	S 162	-	0.840	0.596	0.718	0.173
Q57XV0	Tb927.3.1170	Uncharacterized protein	S 248	0.536	0.984	0.613	0.711	0.240
Q388X2	Tb10.389.0880	Heat shock protein, putative	S 137	0.508	0.702	0.666	0.625	0.103
Q385P1	Tb11.02.2940	Ubiquitin carboxyl-terminal hydrolase, putative	S 9	0.805	0.498	0.462	0.588	0.188
Q583R5	Tb927.6.2690	Ubiquitin carboxyl-terminal hydrolase, putative	S 184	-	0.656	0.465	0.560	0.135
Q57ZH2	Tb927.7.5170	60S ribosomal protein L23a	S 151	0.697	0.586	0.293	0.526	0.209
Q57WH1	Tb927.7.3560	Uncharacterized protein	S 960	0.559	0.471	0.488	0.506	0.047
Q57YN8	Tb927.8.6370	Uncharacterized protein	S 311	-	0.528	0.479	0.503	0.035
Q57YN8	Tb927.8.6370	Uncharacterized protein	S 314	-	0.528	0.479	0.503	0.035
Q38EP6	Tb09.160.5060	Putative uncharacterized protein	T 4	0.146	0.665	0.616	0.476	0.287
Q389U9	Tb10.406.0650	Microtubule-associated protein, putative	S 2053	0.042	0.648	0.513	0.401	0.318
Q586S2	Tb927.2.4780	Uncharacterized protein	S 350	0.139	-0.611	-0.656	-0.376	0.447
Q57WH0	Tb927.7.3550	Uncharacterized protein	S 753	-0.176	-0.582	-0.595	-0.451	0.238
Q57ZR3	Tb927.5.1940	Uncharacterized protein	S 146	-0.697	-0.549	-1.130	-0.792	0.302
Q57ZR3	Tb927.5.1940	Uncharacterized protein	T 152	-0.697	-0.549	-1.130	-0.792	0.302
Q57ZR3	Tb927.5.1940	Uncharacterized protein	S 146	-0.756	-0.617	-1.057	-0.810	0.225
Q386F1	Tb11.02.1100	Nucleobase/nucleoside transporter 8.1	S 240	-	-1.552	-1.225	-1.388	0.231
Q38CW6	Tb09.244.2560	Kinesin-like protein	S 469	-2.078	-	-1.127	-1.603	0.672
Q4FKA6	Tb11.1400	Translation initiation factor eIF2B delta subunit, putative	S 207	-2.470	-2.403	-1.361	-2.078	0.622

Table 2: Significantly regulated phosphorylation sites in procyclic forms of *T. brucei* silenced for MKK5.

References

- 1 Matthews, K. R. The developmental cell biology of *Trypanosoma brucei*. *Journal of cell science* **118**, 283-290, doi:10.1242/jcs.01649 (2005).
- 2 Marchini, F. K., de Godoy, L. M., Batista, M., Kugeratski, F. G. & Krieger, M. A. Towards the phosphoproteome of trypanosomatids. *Sub-cellular biochemistry* **74**, 351-378, doi:10.1007/978-94-007-7305-9_15 (2014).
- 3 Parsons, M., Worthey, E. A., Ward, P. N. & Mottram, J. C. Comparative analysis of the kinomes of three pathogenic trypanosomatids: *Leishmania major*, *Trypanosoma brucei* and *Trypanosoma cruzi*. *BMC genomics* **6**, 127, doi:10.1186/1471-2164-6-127 (2005).
- 4 Qi, M. & Elion, E. A. MAP kinase pathways. *Journal of cell science* **118**, 3569-3572, doi:10.1242/jcs.02470 (2005).
- 5 Songyang, Z. *et al.* A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1. *Molecular and cellular biology* **16**, 6486-6493 (1996).
- 6 Hua, S. B. & Wang, C. C. Interferon-gamma activation of a mitogen-activated protein kinase, KFR1, in the bloodstream form of *Trypanosoma brucei*. *The Journal of biological chemistry* **272**, 10797-10803 (1997).
- 7 Muller, I. B., Domenicali-Pfister, D., Roditi, I. & Vassella, E. Stage-specific requirement of a mitogen-activated protein kinase by *Trypanosoma brucei*. *Molecular biology of the cell* **13**, 3787-3799, doi:10.1091/mbc.E02-02-0093 (2002).
- 8 Ellis, J., Sarkar, M., Hendriks, E. & Matthews, K. A novel ERK-like, CRK-like protein kinase that modulates growth in *Trypanosoma brucei* via an autoregulatory C-terminal extension. *Molecular microbiology* **53**, 1487-1499, doi:10.1111/j.1365-2958.2004.04218.x (2004).
- 9 Domenicali Pfister, D. *et al.* A Mitogen-activated protein kinase controls differentiation of bloodstream forms of *Trypanosoma brucei*. *Eukaryotic cell* **5**, 1126-1135, doi:10.1128/EC.00094-06 (2006).
- 10 Guttinger, A., Schwab, C., Morand, S., Roditi, I. & Vassella, E. A mitogen-activated protein kinase of *Trypanosoma brucei* confers resistance to temperature stress. *Molecular and biochemical parasitology* **153**, 203-206, doi:10.1016/j.molbiopara.2007.02.001 (2007).
- 11 Mackey, Z. B., Koupparis, K., Nishino, M. & McKerrow, J. H. High-throughput analysis of an RNAi library identifies novel kinase targets in *Trypanosoma brucei*. *Chemical biology & drug design* **78**, 454-463, doi:10.1111/j.1747-0285.2011.01156.x (2011).
- 12 Batista, M. *et al.* The MAP kinase MAPK1 is essential to *Trypanosoma brucei* proliferation and regulates proteins involved in mRNA metabolism. *Journal of proteomics* **154**, 118-127, doi:10.1016/j.jprot.2016.12.011 (2017).
- 13 Jensen, B. C., Kifer, C. T. & Parsons, M. *Trypanosoma brucei*: Two mitogen activated protein kinase kinases are dispensable for growth and virulence of the bloodstream form. *Experimental parasitology* **128**, 250-255, doi:10.1016/j.exppara.2011.03.001 (2011).
- 14 Pan, C., Olsen, J. V., Daub, H. & Mann, M. Global effects of kinase inhibitors on signaling networks revealed by quantitative phosphoproteomics. *Molecular & cellular proteomics : MCP* **8**, 2796-2808, doi:10.1074/mcp.M900285-MCP200 (2009).

- 15 Hilger, M., Bonaldi, T., Gnad, F. & Mann, M. Systems-wide analysis of a phosphatase knock-down by quantitative proteomics and phosphoproteomics. *Molecular & cellular proteomics : MCP* **8**, 1908-1920, doi:10.1074/mcp.M800559-MCP200 (2009).
- 16 Bodenmiller, B. *et al.* Phosphoproteomic analysis reveals interconnected system-wide responses to perturbations of kinases and phosphatases in yeast. *Science signaling* **3**, rs4, doi:10.1126/scisignal.2001182 (2010).
- 17 Redmond, S., Vadivelu, J. & Field, M. C. RNAi: an automated web-based tool for the selection of RNAi targets in *Trypanosoma brucei*. *Molecular and biochemical parasitology* **128**, 115-118 (2003).
- 18 Wickstead, B., Ersfeld, K. & Gull, K. Targeting of a tetracycline-inducible expression system to the transcriptionally silent minichromosomes of *Trypanosoma brucei*. *Molecular and biochemical parasitology* **125**, 211-216 (2002).
- 19 Wirtz, E., Leal, S., Ochatt, C. & Cross, G. A. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. *Molecular and biochemical parasitology* **99**, 89-101 (1999).
- 20 Brun, R. & Schonenberger. Cultivation and in vitro cloning or procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Short communication. *Acta tropica* **36**, 289-292 (1979).
- 21 Brenndorfer, M. & Boshart, M. Selection of reference genes for mRNA quantification in *Trypanosoma brucei*. *Molecular and biochemical parasitology* **172**, 52-55, doi:10.1016/j.molbiopara.2010.03.007 (2010).
- 22 Ong, S. E. *et al.* Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Molecular & cellular proteomics : MCP* **1**, 376-386 (2002).
- 23 de Godoy, L. M. *et al.* Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. *Nature* **455**, 1251-1254, doi:10.1038/nature07341 (2008).
- 24 Urbaniak, M. D., Martin, D. M. & Ferguson, M. A. Global quantitative SILAC phosphoproteomics reveals differential phosphorylation is widespread between the procyclic and bloodstream form lifecycle stages of *Trypanosoma brucei*. *Journal of proteome research* **12**, 2233-2244, doi:10.1021/pr400086y (2013).
- 25 Rappsilber, J., Ishihama, Y. & Mann, M. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Analytical chemistry* **75**, 663-670 (2003).
- 26 Rappsilber, J., Mann, M. & Ishihama, Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nature protocols* **2**, 1896-1906, doi:10.1038/nprot.2007.261 (2007).
- 27 Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature biotechnology* **26**, 1367-1372, doi:10.1038/nbt.1511 (2008).
- 28 Cox, J. *et al.* Andromeda: a peptide search engine integrated into the MaxQuant environment. *Journal of proteome research* **10**, 1794-1805, doi:10.1021/pr101065j (2011).
- 29 Tyanova, S. *et al.* The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nature methods* **13**, 731-740, doi:10.1038/nmeth.3901 (2016).
- 30 Szklarczyk, D. *et al.* STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic acids research* **43**, D447-452, doi:10.1093/nar/gku1003 (2015).

- 1
2
3 31 Cox, J. & Mann, M. 1D and 2D annotation enrichment: a statistical method
4 integrating quantitative proteomics with complementary high-throughput data. *BMC*
5 *bioinformatics* **13 Suppl 16**, S12, doi:10.1186/1471-2105-13-S16-S12 (2012).
- 6 32 Kimball, S. R., Fabian, J. R., Pavitt, G. D., Hinnebusch, A. G. & Jefferson, L. S.
7 Regulation of guanine nucleotide exchange through phosphorylation of eukaryotic
8 initiation factor eIF2 α . Role of the α - and δ -subunits of eIF2b. *The*
9 *Journal of biological chemistry* **273**, 12841-12845 (1998).
- 10 33 Jennings, M. D. & Pavitt, G. D. A new function and complexity for protein translation
11 initiation factor eIF2B. *Cell cycle* **13**, 2660-2665, doi:10.4161/15384101.2014.948797
12 (2014).
- 13 34 Lee, S. H., Stephens, J. L. & Englund, P. T. A fatty-acid synthesis mechanism
14 specialized for parasitism. *Nature reviews. Microbiology* **5**, 287-297,
15 doi:10.1038/nrmicro1617 (2007).
- 16 35 Lee, S. H., Stephens, J. L., Paul, K. S. & Englund, P. T. Fatty acid synthesis by
17 elongases in trypanosomes. *Cell* **126**, 691-699, doi:10.1016/j.cell.2006.06.045 (2006).
- 18 36 Bringaud, F., Riviere, L. & Coustou, V. Energy metabolism of trypanosomatids:
19 adaptation to available carbon sources. *Molecular and biochemical parasitology* **149**,
20 1-9, doi:10.1016/j.molbiopara.2006.03.017 (2006).
- 21 37 Bochud-Allemann, N. & Schneider, A. Mitochondrial substrate level phosphorylation
22 is essential for growth of procyclic *Trypanosoma brucei*. *The Journal of biological*
23 *chemistry* **277**, 32849-32854, doi:10.1074/jbc.M205776200 (2002).
- 24 38 Besteiro, S., Barrett, M. P., Riviere, L. & Bringaud, F. Energy generation in insect
25 stages of *Trypanosoma brucei*: metabolism in flux. *Trends in parasitology* **21**, 185-
26 191, doi:10.1016/j.pt.2005.02.008 (2005).
- 27 39 van Hellemond, J. J., Opperdoes, F. R. & Tielens, A. G. The extraordinary
28 mitochondrion and unusual citric acid cycle in *Trypanosoma brucei*. *Biochemical*
29 *Society transactions* **33**, 967-971, doi:10.1042/BST20050967 (2005).
- 30 40 van Weelden, S. W., van Hellemond, J. J., Opperdoes, F. R. & Tielens, A. G. New
31 functions for parts of the Krebs cycle in procyclic *Trypanosoma brucei*, a cycle not
32 operating as a cycle. *The Journal of biological chemistry* **280**, 12451-12460,
33 doi:10.1074/jbc.M412447200 (2005).
- 34 41 Van Hellemond, J. J., Opperdoes, F. R. & Tielens, A. G. Trypanosomatidae produce
35 acetate via a mitochondrial acetate:succinate CoA transferase. *Proceedings of the*
36 *National Academy of Sciences of the United States of America* **95**, 3036-3041 (1998).
- 37 42 Korotchkina, L. G. & Patel, M. S. Mutagenesis studies of the phosphorylation sites of
38 recombinant human pyruvate dehydrogenase. Site-specific regulation. *The Journal of*
39 *biological chemistry* **270**, 14297-14304 (1995).
- 40 43 Patel, M. S. & Korotchkina, L. G. Regulation of the pyruvate dehydrogenase
41 complex. *Biochemical Society transactions* **34**, 217-222, doi:10.1042/BST20060217
42 (2006).
- 43 44 Nett, I. R. *et al.* The phosphoproteome of bloodstream form *Trypanosoma brucei*,
44 causative agent of African sleeping sickness. *Molecular & cellular proteomics : MCP*
45 **8**, 1527-1538, doi:10.1074/mcp.M800556-MCP200 (2009).
- 46 45 John von Freyend, S. *et al.* LmxMPK4, an essential mitogen-activated protein kinase
47 of *Leishmania mexicana* is phosphorylated and activated by the STE7-like protein
48 kinase LmxMKK5. *International journal for parasitology* **40**, 969-978,
49 doi:10.1016/j.ijpara.2010.02.004 (2010).
- 50 46 Erdmann, M., Scholz, A., Melzer, I. M., Schmetz, C. & Wiese, M. Interacting protein
51 kinases involved in the regulation of flagellar length. *Molecular biology of the cell* **17**,
52 2035-2045, doi:10.1091/mbc.E05-10-0976 (2006).
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55
56
57
58
59
60

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